Temporal Changes in Microbial Community Composition and Geochemistry in Flowback and Produced Water from the Duvernay Formation

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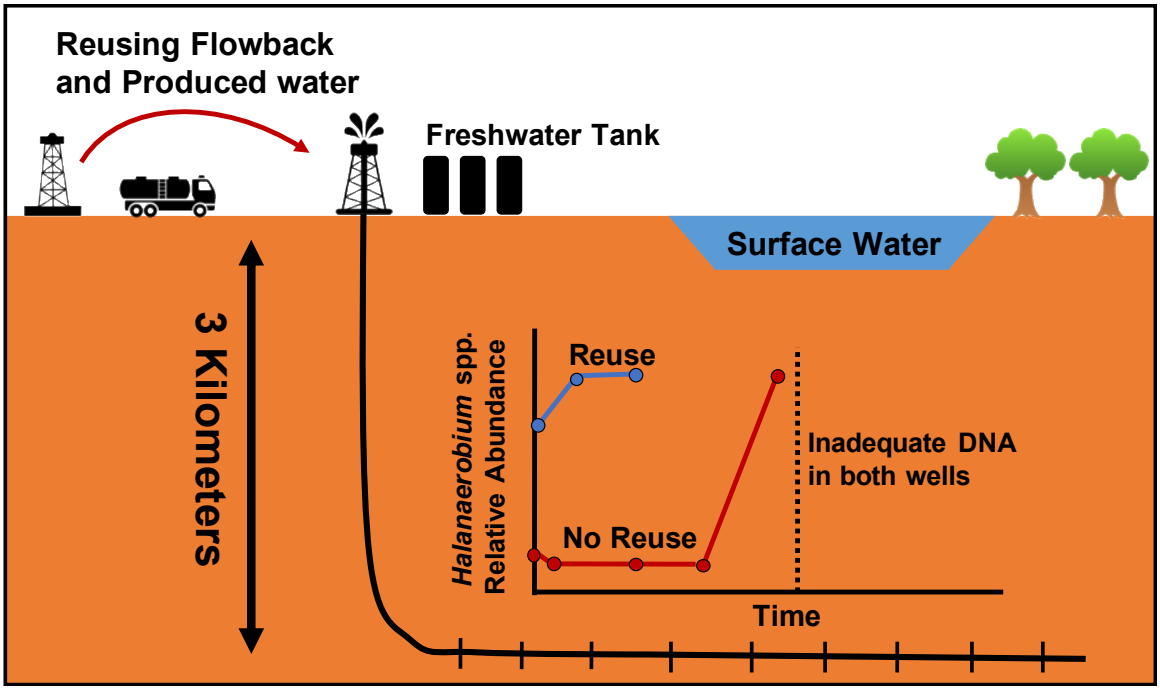
Abstract

Microbial activity in flowback and produced water (FPW) may negatively influence shale oil and gas extraction, however the impacts of using recycled produced water (RPW) for subsequent fracturing jobs are not well-understood. In this study, we compared time series of FPW samples from two horizontally fractured wells drilled into the Duvernay Formation in Alberta, Canada, well 1 used RPW in the makeup of the hydraulic fracturing fluid (HFF) while well 2 did not. 16S rRNA gene sequencing and live/dead cell enumeration were used to track microbial communities. Within 20 days of flowback, total dissolved solids in well 1 and well 2 increased from 5,310 mg/L and 288 mg/L to over 150,000 mg/L, and FPW temperatures increased from 20°C and 9°C to 77°C and 71°C, respectively. Alkyl dimethyl benzyl ammonium chloride (biocide) in well 2 decreased from 25 µg/L to below the detection limit of 0.5 µg/L. Cellular biomass decreased from ~10^5 cells mL^{-1} to less than the detection limit of 10^5 cells mL^{-1} in both wells, and the community in the samples was initially diverse, but rapidly shifted to become dominated by the sulfidogenic lineage *Halanaerobium*. Methanogens were detected at low relative abundance within archaea. DNA concentrations in FPW after 20 days were inadequate for sequencing. Comparing the two wells, the start time of *Halanaerobium* enrichment was considerably shortened in well 1 relative to well 2. Our results suggest that subsurface environmental parameters primarily drive the rapid enrichment of sulfidogenic and halotolerant bacteria and current recycling strategies can facilitate the growth of these bacteria, while biocide seems to be a less important factor in this shift.



Keywords

Microbial communities, geochemistry, flowback and produced water, recycling strategies, Duvernay Formation, shale oil and gas

1. Introduction

Horizontal drilling combined with hydraulic fracturing has been increasingly used to recover oil and gas from tight geologic formations by creating extensive fracture networks.[1–3] This process is water intensive, with the average volume of water used for fracturing estimated to be 15,000 m^3 per well.[4,5] To prepare hydraulic fracturing fluids, a wide variety of chemicals and proppants (e.g. sand) are added to water, most commonly freshwater. Not only is surface freshwater consumed by hydraulic fracturing, but a significant amount of wastewater is also produced, as once fracturing is complete, pressure is released at the wellhead, resulting in the

return of the fraction fracturing fluid as flowback and produced water (FPW) to the surface.[6] The FPW typically contains components of the original fracturing fluids, dissolved constituents from shale formations, and reaction byproducts formed under the elevated subsurface temperatures and pressures in the well.[5,7,8] FPW components, such as salts and organic contaminants, can have detrimental effects on aquatic organisms if not treated properly.[9–17] The most common practice for FPW disposal is deep well injection, but this strategy may permanently remove water resources from the surface environment.[18] Recycling or partially reusing FPW to make hydraulic fracturing fluids has been regarded as a potential approach to partially mitigate the water consumption by hydraulic fracturing operations.[3] Current recycling strategies involve mixing pre-treated FPW to freshwater source water as hydraulic fracturing fluids, with the specific mixing ratio varying between operators, in part depending upon differing geochemistry.

Microbial activities have potential to promote the souring of gas, facilitate corrosion, biofouling of the well and drilling equipment, and biodegradation of components fracturing fluid components such as guar gum.[19–23] Substrates from hydraulic fracturing additives can cause microorganisms, in particular halotolerant bacteria, to persist in the FPW.[24] The use of biocides to mitigate these detrimental effects from microorganisms are inefficient, for biocides are likely diluted, degraded, and/or transformed into other compounds under downhole conditions.[25–27] Additionally, when the FPW returns to the surface, microbial communities in the FPW can lead to metabolisms that may result in the release of greenhouse

gases,[28,29] and other undesirable gases including H_2S to the atmosphere. Reuse or recycling of FPW in making up new hydraulic fracturing fluid (HFF) increases the salinity of that fluid, and thus may seed HFF with microbes that are tolerant of the conditions found in the subsurface, causing either more rapid or complete development of detrimental microbial communities.[30,31] Within the halotolerant bacteria, *Halanaerobium* are one of the most prevalent microorganisms found in hydraulic fracturing sites such as Marcellus Formation, Pennsylvania.[24,25,30–35] Species of *Halanaerobium* have the potential to reduce thiosulfate to sulfide,[22] and therefore they are important in evaluating geochemical processes at downhole conditions in hydraulic fracturing sites. Previous studies demonstrated that *Halanaerobium* species are prevalent in late-stage FPW.[32] However, most of those studies focus on U.S. shale plays, with few publications that have investigated shale formations in other locations that may have different geochemistry and microbiology. The Duvernay Formation (Upper Devonian) is one of the largest shale oil and gas fields in Canada, deposited in the over three kilometers deep subsurface and surrounded by the Leduc reefs complex (Figure S1).[36] Compared to other shale reservoirs, the Duvernay Formation was deposited in a relatively closed marine system with significant calcareous components.[36,37] To the best of our knowledge, the microbial community composition during well flowback of the Duvernay Formation has yet to be studied.

In this study, we characterized the microbial cellular biomass, viability, community composition and diversity in FPW as a function of flowback time (up to 120 days)

from fracturing of two unconventional wells in Duvernay Formation using 16S rRNA sequencing and live/dead cell staining. We linked shifts in community composition to the changes in FPW geochemistry and temperatures based on a wide range of chemical tracers, including pH, total dissolved solids (TDS), organic carbon content and composition, and major cations and anions. Finally, we compared the shift in microbial community composition between two wells, one of which used freshwater only as the base of the fracking fluid and the other which used recycled FPW and freshwater mix. Our results provide new understanding of reasons for differences in the microbiology of FPW related to the recycling and reuse of produced fluids in making up injected HFF.

2. Materials and Methods

2.1 Sampling

Two horizontally drilled and hydraulically fractured wells of the Duvernay Formation belonging to the Encana Corporation were sampled in the Fox Creek region, Alberta, Canada (Figure 1). Well 1 reused FPW in combination with fresh surface water to make up the hydraulic fracturing fluid used to fracture the well, while in well 2 only freshwater was used. The rig release date for the well 1 was at 2016-07-08 and the flowback started at 2016-09-09 (63 days later), and the rig release date for the well 2 was at 2016-09-21 and the flowback started at 2016-12-05 (75 days later). The FPW samples were collected by Encana personnel from the two wells starting at the beginning of the flowback process and continuing for 120 days for well 1 and 102 days for well 2 afterward, respectively. FPW samples

from well 1 were collected between August 2016 and January 2017, and from well 2 between December 2016 and March 2017 (the sampling frequency is presented in Table S1). The source water (before adding chemical additives and recycled produced water) used to make up of hydraulic fracturing fluids was taken from a nearby river and collected at water storage ponds near well 1 and well 2. For well 1, FPW samples were collected passing through the oil/water separator, while for well 2, FPW samples were collected at the wellhead before returning fluids reaching the oil/water separator, because multiple wells were flowing into the same oil/water separator; in this way, it was ensured that all collected return fluids originated from well 2 only on that pad. Fluid samples were collected in 20 L polyethylene pails for DNA extraction, and 2 L amber glass bottles for chemistry analyses. The 20 L polyethylene pails were new and kept dry condition until use. Before collecting FPW samples, the pails were pre-rinsed with the FPW to be sampled. The sealed containers were transported to University of Alberta within 24-72 hours. Cell staining, cell collection, and DNA extraction were done within 48 hours of sample arrival.

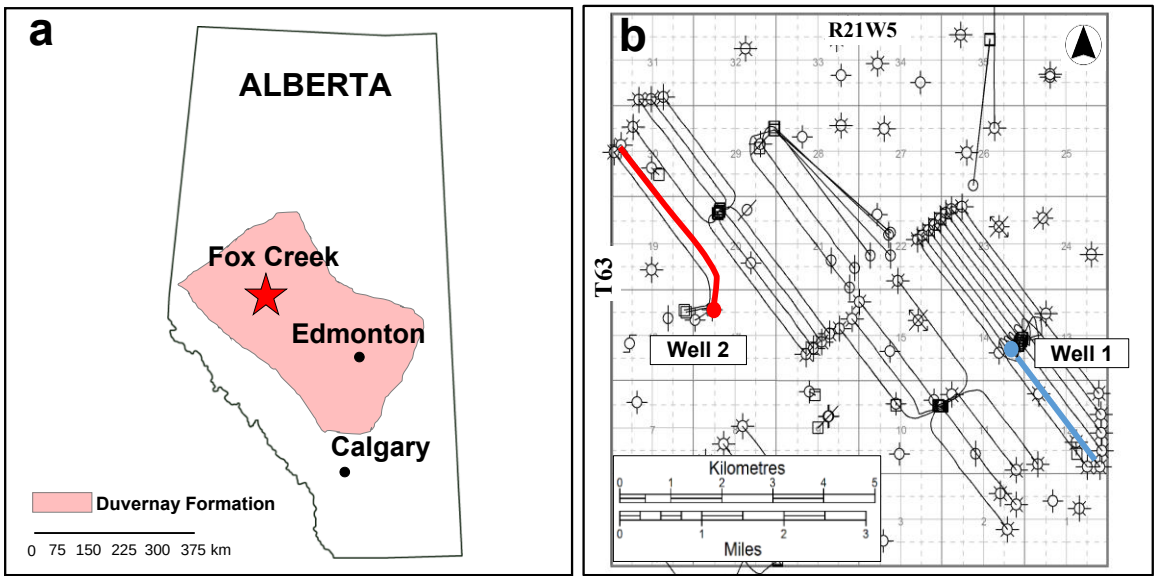


Figure 1 (a) Map of Alberta indicating the location of the Fox Creek region, where the sampled wells are located, and (b) a local-scale map showing the locations of wells 1 and 2, modified from geoSCOUT

2.2 Fluid temperature and geochemistry analyses

Temperature, pH, TDS, major ions, and organic components were measured to evaluate the influence of change in environmental parameters on the microbial communities. H_2S concentrations and fluid temperature were measured by site personnel on the wellpad once the FPW returned to the surface. The TDS concentrations of samples were determined by evaporating 10 mL of FPW at 200ºC. Prior to cation, anion, and total organic carbon (TOC) analyses, fluid samples were filtered through 0.2 µm nylon membranes (Agilent Technologies, Ontario, Canada). Cations, bromide and total sulfur concentrations were measured using an Agilent 8800 inductively coupled plasma - mass spectrometer (Agilent Technologies, California, USA). Anion concentrations were measured using a DX

600 ion chromatograph (Dionex, California, USA). TOC was measured as non-purgeable organic carbon using the TOC-V CHS/CSN Analyzer (Shimadzu, Kyoto, Japan). FPW samples were filtered through 0.45 µm PTFE membranes before analysis of organic compounds by high performance liquid chromatography (HPLC) coupled with ultra-high resolution Orbitrap mass spectrometry (MS) using positive electrospray ionization (ESI^+) (Thermo Fisher Scientific, Massachusetts, USA). Additional analytical details on the FPW chemistry are given in the Supporting Information.

2.3 Enumeration of live and dead cells

To assess bacterial viability, the Live/Dead BacLight Viability kit (Life Technologies, Ontario, Canada) was used according to the manufacturer's instructions. Cell enumeration was performed within 24 hours of the arrival of samples to minimize changes in the live/dead ratio and cell counts. To prepare for cell counting, 250 µL of a sample was mixed with 250 µL staining dye that consisted of 6 µM SYTO 9 stain and 30 µM propidium iodide. Samples were incubated in the dark for 15 min at room temperature, then filtered onto 0.2 µm black polycarbonate membranes (GE Healthcare Life Sciences, Ontario, Canada) with Whatman GF-F glass microfiber filters (GE Healthcare Life Sciences, Ontario, Canada) used as membrane support filters. For each sample, 15 randomly selected fields were counted at 358× magnification on a Leica DMRXA epifluorescence microscope equipped with FITC ("live") and Rhodamine ("dead") fluorescence filters for the estimation of total cell numbers (the sum of live and dead cells) and cell viability

(the proportion of live cells). The detection limit was 10^5 cells mL^{-1} (total cell numbers less than 200 in total 15 microscope fields). A t-test was used to determine the significant difference of the total cell numbers between each time point.

2.4 DNA Extraction and 16S rRNA gene sequencing

For 16S rRNA gene sequencing, 500-1500 mL of each water sample was filtered through 0.22 µm pore size hydrophilic polypropylene membranes (GE Healthcare Life Sciences, Ontario, Canada) within 24h of sample arrival and then stored at -80°C until further processing. The membrane filters were sterile and were connected to a filtration manifold. The filtration glassware was rinsed with distilled and deionized water and then dried at 150°C for 30 min prior to filtrations. These filters were then cut into small pieces using a sterile scalpel and DNA was extracted from the filters using the FastDNA spin kit for soil following the manufacturer's instructions (MP Biomedicals, Solon, USA). Further details on DNA extraction are provided in the Supporting Information. 16S rRNA gene fragments covering the V4 variable region of the gene were amplified using the universal Bacteria and Archaea primers F515 (5′- GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACHVGGGTWTCTAAT-3′).[38] Polymerase chain reaction (PCR) was conducted using KAPA HiFi HotStart Ready Mix (Fisher scientific, Ontario, Canada). The PCR reaction began with a 3 min initial denaturation (95°C) followed by 35 cycles of 30 sec denaturation (95°C), 30 sec primer annealing (55°C), and 30 sec extension (72°C) and a final 5 min extension (72°C). Duplicate PCR

reactions were combined prior to library construction. Paired end sequencing was conducted on an Illumina MiSeq System at The Applied Genomics Core Sequencing Facility at the University of Alberta.

2.5 Bioinformatics and statistical analyses

Raw sequences for each sample from Illumina MiSeq were quality filtered, and aligned to a combined bacterial and archaeal SILVA database (version 128) using Mothur software (version 1.39.5).[39,40] Chimeras were removed using UCHIME.[41] Operational taxonomic units (OTUs; sequences ≥97% similarity) were classified according to the highest taxonomic resolution possible using Bayesian classifier with an 80% confidence threshold (chloroplast, mitochondria, and Eukaryota removed).[42] Subsequently, each library was normalized to 3154 sequences, the smallest library size.[43] For the alpha-diversity analyses, the Good's coverage estimator, Chao1 richness estimator, non-parametric Shannon diversity index, and inverse Simpson diversity index as implemented in Mothur (version 1.39.5) were used to analyze the microbial community coverage, richness, and diversity, respectively, of each library.[44–47] For beta-diversity analyses, the Bray-Curtis distance between samples were shown on the Canonical Analysis of Principle coordinates (CAP) using phyloseq package in R software (3.5.1).[48] ANOVA and PERMANOVA statistical analyses were used to determine the statistical significance of the correlation between environmental variables and microbial community dissimilarities for each sample library on the CAP ordination. Spearman correlation estimated the influence of OTUs to the sample dissimilarities on the

CAP ordination. We also reconstructed the maximum likelihood phylogenetic tree of *Halanaerobium* spp. 16S rRNA genes in order to compare the similarity of *Halanaerobium* sequences detected in our study to other *Halanaerobium* sequences detected from other places. The detailed methods of maximum likelihood phylogenetic tree reconstruction are presented in the Supporting Information. Sequences have been uploaded to the Sequence Read Archive (BioProject: PRJNA407226).

3. Results and Discussion

3.1 temperature pH, and salinity of FPW from the Duvernay Formation

The microbial community structure in FPW can be dramatically influenced by environmental variables.[31,34] For example, high temperatures in the Bakken Formation are thought to naturally limit the growth of microorganisms in FPW.[49] The Duvernay Formation is located over three kilometers into deep subsurface, and the reservoir temperature is estimated at 115°C in the targeted region,[50] close to the 122°C upper threshold of growth temperature for any known organism.[51] FPW temperatures for both wells were measured at the wellhead immediately when fluids returned to the surface. The fluid temperatures recorded at each wellhead were similar; well 1 FPW increased from 20°C initially to its highest value of 77°C by day 4, and well 2 FPW gradually increased from 9°C initially to the maximum value of 71°C by day 10 (Figure 2A). After reaching the maximum values, the FPW temperatures for both wells dropped and were then consistently in the range of 50-60°C. Due to the geothermal gradient, the temperature of the FPW

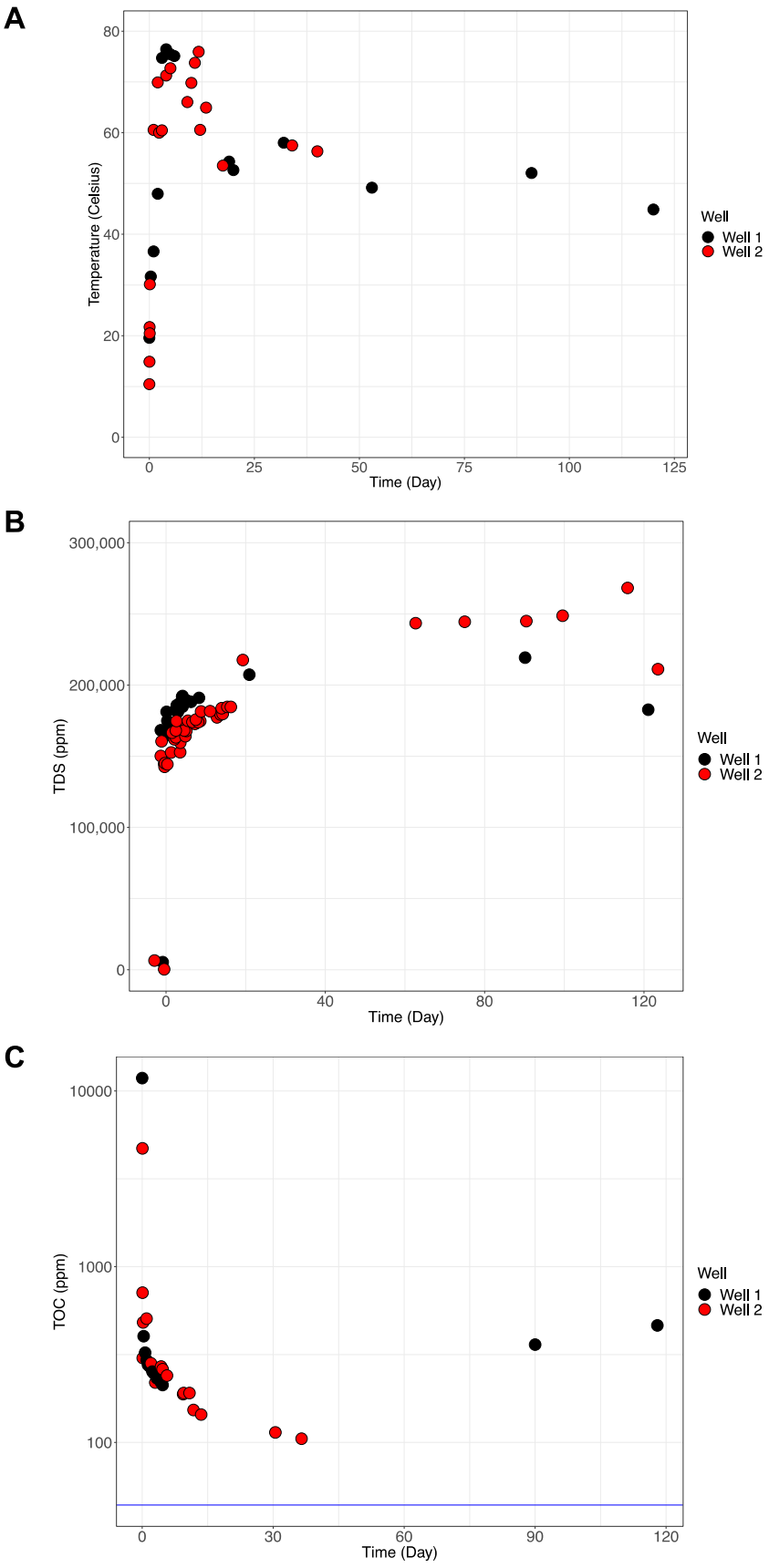
may decrease as fluid travels through higher, cooler stratigraphic layers. Thus, the actual FPW temperatures downhole are likely higher than what were measured at the wellhead. FPW pH declined modestly as flowback proceeded. The pH decreased from slightly basic in the injected source water (well 1: pH 8.1, well 2: pH 7.4), to slightly acidic conditions in the FPW samples after the initial FPW commenced, which were consistently in the range of 5.2-6.1 (Table S2), and are within the range of the FPW samples collected from other shale gas extraction sites.[27] TDS of source water was 332 parts per million (ppm). For both wells, a rapid and large increase in TDS from 5310 ppm for well 1 initial FPW and 288 ppm for well 2 initial FPW to >150,000 ppm within a day of the start of well flowback. TDS reached a peak of 219,000 ppm by day 53 for well 1 and 268,000 ppm by day 115 for well 2 (Figure 2B). The higher TDS for well 1 at the start of well flowback, primarily composed of sodium and chloride (Table S3), is likely due to the reuse of FPW from another well as part of the composition of hydraulic fracturing fluids. The fast rate of increase in TDS was likely linked to the long shut-in time of both wells (i.e. the gap between the time of rig release and the start time of flowback), and/or the influx of formation brine and/or leaching of formation minerals.[52,53] The presence of calcareous mudstones and carbonates in the Duvernay Formation are likely to result in the presence of naturally fractured rock and connate water, which may contribute to the fast rise of salinity in FPW.[54] The previous study found that the Day 1 FPW sample from well 1 had 254 ppm Fe(II),[55] indicating anoxic conditions in FPW from the Duvernay Formation. Compared to the low content of iron and sulfur in source water (Table S3), these substantial elements in FPW can

offer more growth potential for microbes, which need electron acceptors under anoxic conditions.

3.2 Major organic compounds of aqueous phase

For both wells, the TOC concentrations in the first FPW samples were the highest; 11,800 ppm and 4,710 ppm for wells 1 and 2, respectively, which then fell to within ranges of 200-500 ppm and 100-500 ppm within the 120 days of the start of flowback (Figure 2C). The TOC contents of these FPW samples were much higher than that of source water. The total ion chromatograms showed the ion intensity of the FPW samples from well 2 decreased 84% from 1.44 h to day 3 after well flowback, indicating the dissolved organic concentrations were reduced with increasing time of flowback (Figure 3). A series of polyethylene glycol (PEG) compounds, octylpenol ethoxylates (OPE) and alkyl dimethyl benzyl ammonium chloride (ADBAC) were identified as major organic compounds in the FPW samples from Duvernay Formation (Figure 3). The PEGs are surfactants used in hydraulic fracturing fluids, and low molecular weight PEGs can serve as carbon sources for microbial communities.[56,57] ADBAC can be visually observed on the extracted ion chromatograms (Figure S2). The ADBAC homologues ranging from C8 to C14, decreased from 25 parts per billion (ppb) at 1.44 hours, to below the detection limit of 0.5 ppb by day 3 of well flowback. The ADBAC is one of the main lytic biocides used to inhibit the growth of microbial communities,[26] which was the only biocide detected in the Duvernay FPW samples. The high TOC content combined with the individual organic compounds suggest that a considerable

amount of organic carbon was transported to the subsurface through the injection of hydraulic fracturing fluids. The identification details of the organic compounds in the well 2 FPW samples can be found in the Supporting Information.


A
Temperature (Celsius)
Time (Day)
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B
TDS (ppm)
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Well
Well 1
Well 2
C
TOC (ppm)
Time (Day)
Well
Well 1
Well 2

Figure 2 Temporal changes in (A) FPW flow temperatures, (B) FPW TDS, (C) TOC concentrations in FPW for the two wells, all plotted as time since initiation of well flowback (44 ppm TOC concentration of the source water for well 1 is shown as the blue horizontal line). Well 1 used RPW for hydraulic fracturing.

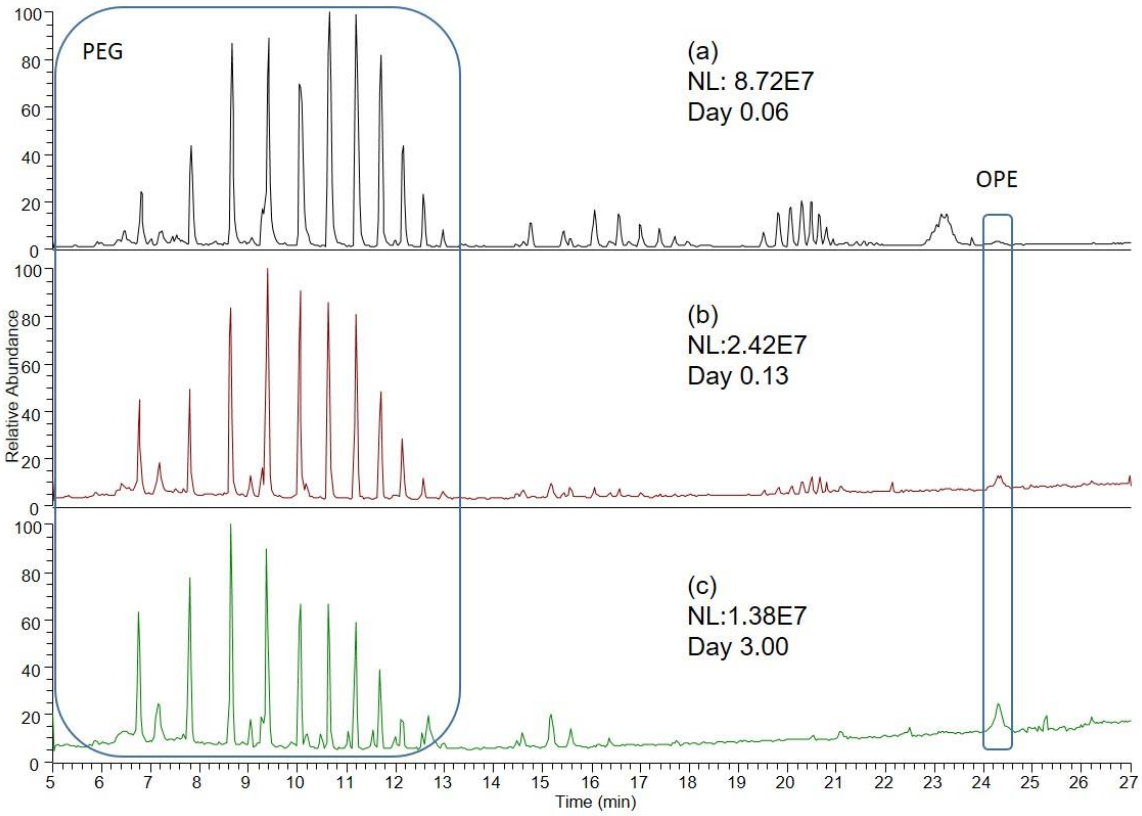

Figure 3 HPLC-Orbitrap MS total ion chromatograms of FPW samples collected from Well 2 at (a) Day 0.06, (b) Day 0.13 and (c) Day 3 after initiation of flowback. NL is the normalized total ion abundance.

3.3 Total cell numbers and live cell proportion

Cell populations were assessed by observing the change of total cell numbers and cell viability. For both wells, the overall trend was a reduction of total cell numbers

from 10^6 cells mL^{-1} in the source water, to 10^5 cells mL^{-1} in the early-stage FPW (Table 1), and then to less than 10^5 cells mL^{-1} in late-stage FPW. A significant difference ($p<0.05$) of the total cell numbers were determined by comparing the source water to the initial FPW. With ongoing flowback, the total cell numbers in the well 1 FPW significantly decreased from $0.72\pm0.13 \times 10^6$ cells mL^{-1} by 8 hours to $0.15\pm0.11 \times 10^6$ cells mL^{-1} by day 1; the total cell numbers in the well 2 FPW were also significantly reduced from $0.40\pm0.29 \times 10^6$ cells mL^{-1} at 1 hour to $0.40\pm0.29 \times 10^6$ cells mL^{-1} by day 1, and then further decreased to $0.10\pm0.07 \times 10^6$ cells mL^{-1} by day 10. As time of flowback increased (>10 days) in both wells, when geochemistry of the FPW dramatically differs from original hydraulic fracturing fluids, the total cell numbers were reduced to below the detection limit (below or near to 10^5 cell mL^{-1}) and did not recover within the over three-month observation (Table S4). The decrease of the total biomass is likely due to the extreme downhole conditions. Similar to the present studies, low cellular biomass was observed by microscopy of FPW collected from the Marcellus and Burket shale oil and gas fields in Pennsylvania.[29]

In addition to the total cell abundance, we also assessed the viability of cells in FPW. For the two investigated wells, the overall viability of cells was higher in the initial FPW and decreased as a function of return time (Table 1). In contrast to the trend observed for the total cell numbers, we did not observe a steep reduction in the percent of viable cells from the source water relative to the initial FPW, and subsequent flowback over time. The approximately 40% of cells were viable

(based on the criterion of having an intact cell membrane) in the initial FPW samples for both wells; this ratio decreased to the lowest value of 7% by day 2 for well 1 and 12% by day 10 for well 2 (Table S4). The change in the percentage of viable cells suggests a fraction of microorganisms remain viable in the very early-stage FPW.

Table 1 Coverage, richness, diversity, cell numbers, and cell viability of microbial communities for source water and FPW

Well	Type	Time (Day)	Good's Coverage Percentage	Observed OTU	Chao1	Inverse Simpson	Nonparametric Shannon	Total Cell Numbers (10^6 cells mL^{-1}, n=15)	Cell Viability (%)
Well 1	Source Water		99	120	148	20.05	3.56	2.58±0.10	46
	FPW	0	99	40	62	2.30	1.12	0.79±0.19	38
		0.33	100	15	23	1.46	0.64	0.72±0.13	20
		1	100	16	37	1.46	0.66	0.15±0.11	20
Well 2	Source Water		99	116	148	21.36	3.60	1.04±0.30	23
	FPW	0	98	173	246	15.76	3.58	0.48±0.14	40
		0.04	98	142	190	2.34	1.96	0.72±0.19	43
		1	99	86	109	1.90	1.46	0.40±0.29	35
		10	100	47	58	1.65	1.03	0.10±0.07	12
		18	100	21	23	1.16	0.39	0.13±0.07	33

3.4 Shifts of microbial community compositions and diversity

Co-incident with the decrease of cell numbers and viability, alpha-diversity of the microbial communities, including the observed OTU richness, Chao1 richness, Simpson diversity, and Shannon diversity, also decreased (Table 1). ~~Decrease of these indices suggest richness and diversity of these species dramatically decreased over time.~~ The overall co-effect of the environmental parameters (pH, TOC, temperatures, and salinity) was significantly ($p<0.05$) associated with the shift of microbial community composition. Furthermore, each individual environmental variable tested, including TOC, temperature, and salinity, was individually significantly ($p<0.05$) correlated with microbial community shifts.

We further explored the link between the microbial community compositions and the FPW geochemistry and temperature by taxonomic analyses (Figure 4). In well 1, the initial FPW was dominated (96% of total sequences) by the class Clostridia. Within Clostridia, sequences were highly similar to the genera *Halanaerobium* (52% of total sequences), *Selenihalanaerobacter* (40% of total sequences), and *Orenia* (3% of total sequences). These genera are all composed of halotolerant or halophilic microorganisms.[58–60] Subsequently, sequences classified to the genus *Halanaerobium* increased to 82% in the 8 hours FPW sample and 81% in the day 1 FPW sample. The genus *Halanaerobium* is detected in the high saline environment such as oil and gas reservoirs and salt-rich lakes.[61,62] The maximum likelihood phylogenetic tree of *Halanaerobium* spp. 16S rRNA genes showed the genus *Halanaerobium* are likely to be ubiquitous in hydraulic fracturing sites (Figure S3). Two unique sequences (OTU2 and OTU3) related to *Halanaerobium*

were detected in this study. OTU2 is almost indistinguishable from one strain sequenced from the FPW from Marcellus Formation,[34] and OTU3 is also indistinguishable from a strain from Pennsylvania shale gas produced water.[29] Furthermore, they are highly similar to *Halanaerobium* found in other oil and gas wells from around the world, including China, Germany, Netherlands, Russia, and other places (Figure S3). The *Halanaerobium* in the investigated shale gas plays, which are primarily located in the U.S., are most likely to be associated with the isolates *Halanaerobium saccharolyticum* and *Halanaerobium congolense*.[32] Based on the physiology of cultivated members of the genus, *Halanaerobium* are anaerobic, thiosulfate-reducing, halophilic, and moderately thermophilic bacteria.[22,61] Assuming the *Halanaerobium* in the Duvernay have similar metabolism to those found in other sites, *Halanaerobium* strains have the potential to produce sulfide and organic acids in shale oil and gas wells, and polysaccharide polymers, among the chemical additives in injected fluids, are possible substrates for them to grow in the subsurface.[63] Our results demonstrate that *Halanaerobium* might be enriched in the relatively early flowback period, especially they became dominant in the initial flowback of the well 1. In the early-stage FPW, the elevated salinity favors these halotolerant/halophilic microorganisms. Halotolerant bacteria may utilize the injected chemicals such as glycine betaine to produce osmoprotectants in order to survive in the saline environment.[64] The prevalence of these strictly anaerobic *Halanaerobium*, indicating that certain sulfur species (Table S3) might be the thiosulfate, which may serve as electron acceptors to support them at downhole conditions.[22] However, we did not speciate the sulfur compounds observed in these wells. The beta-diversity analyses revealed two different shift pathways, showing dramatically different microbial community

compositions in the initial FPW samples from two wells, which, however, eventually became highly similar over time (Figure 5). For well 2, the shift to predominantly halotolerant bacteria, including the *Halanaerobium,* was delayed relative to well 1 by over two weeks. The initial FPW from well 2 still contained a greater fraction of microbial classes that were also found in the corresponding source water (Figure 4). In addition, 35% of the total sequences were classified as classes Clostridia and Bacilli. Within the Clostridia, sequences similar to the genera *Halanaerobium* (5% of the total sequences) and *Orenia* (8% of the total sequences) were most prevalent. Within the Bacilli, sequences related to the genus *Trichococcus* comprised the largest fraction (15% of total sequences), some species of which are known to form long chains involved in bulking in activated sludge systems.[65] Eventually, a shift to a predominance of *Halanaerobium* (93% of total sequences) occurred by day 18 of well flowback. Spearman analysis showed that *Halanaerobium*, *Orenia*, and *Selenihalanaerobacter* was significantly correlated to the shift in microbial community compositions for the two wells by 20 days of flowback (the Spearman correlation between top 20 OTUs and CAP ordination is presented in Table S5).

The faster enrichment of the halotolerant bacteria in well 1 is probably linked to the recycling strategy prior to fracturing. Reusing FPW for the makeup of hydraulic fracturing fluids may promote the growth of halotolerant bacteria by seeding adapted microorganisms in the FPW to newly-prepared hydraulic fracturing fluids, meanwhile creating an initially saline environment in the hydraulic fracturing and therefore subsequently influencing the early-stage FPW. Thus, the starting time of the enrichment

of halotolerant/halophilic bacteria may be shortened due to the reuse of FPW for fracturing. Likely due to low amounts of DNA, we were not able to amplify 16S rRNA genes after the Day 1 flowback for well 1 and after day 18 FPW for well 2. For the Duvernay Formation, whether or not injected fracturing fluid eventually reaches the reservoir temperature, FPW temperatures recorded at the wellhead considerably exceed those favorable for the growth of a microbial community dominated by *Halanaerobium*, where characterized isolates are known to grow between 20°C and 51°C, after day 3 of flowback from well 1 and after day 1 of flowback for well 2 (Figure 2A).[61,66]

Methanogenesis carried out by Archaea is an important microbial metabolism in energy extraction because it may enhance resource recovery.[67] In this study, a lower relative abundance (<3% of total reads) was observed for Archaea compared to Bacteria, but the change of archaeal community composition also reflected elevated salinity and temperature. For example, sequences classified as *Methanothermococcus* were observed at 0.17% and 0.05% well 1 initial FPW and after 1h FPW of well 2. *Methanothermococcus* have been isolated from deep-sea hydrothermal vent system with growth temperatures between 40°C - 75°C.[68] The growth temperature for *Methanothermococcus* is consistent with the fluid temperature recorded at the wellhead (Figure 2A). In addition, 0.09% and 0.03% of the sequences were classified as *Methanohalophilus* in well 1 day 1 FPW and the well 2 initial FPW. Strains of genus has previously been isolated from the salty lakes [69] and hydraulic fracturing wells.[70] Strains of both *Methanothermococcus* and *Methanohalophilus* are likely to be methanogens; for example, *Methanothermococcus okinawensis* can use hydrogen and carbon dioxide as

energy and carbon sources,[68] and *Methanohalophilus mahii* can use methanol and methylamines.[69] Methanogens at low abundance and growing together with sulfide-producing bacteria were also observed in the Marcellus and Utica shales, where they may consume metabolites such as trimethylamine produced by other microorganisms in FPW.[24]

The injection of the hydraulic fracturing fluid may transport dissolved oxygen to the subsurface. Oxygen content is an important influencing factor on the observed shift of microbial communities. The oxygen has been characterized to be depleted quickly in FPW from Duvernay Formation, probably because the oxygen was consumed by the sulfide oxidation of the pyrite (FeS_2) at downhole conditions.[55] This anoxia is consistent with the microbial shift, from the diverse freshwater microbial community to the predominantly anaerobic bacteria in the FPW (Figure 4). Of note, in well 2, the fraction of sequences similar to the class Betaproteobacteria increased, forming the dominant bacterial class in FPW samples by 1 hour, day 1, and day 10 (Figure 4). Within this class, the dominant sequences were related to the genera *Ralstonia* and *Burkholderia*, which may degrade hydrocarbons. The *Ralstonia* are known to metabolize diesel fuel in saline environments and to aerobically degrade various other hydrocarbons.[71–73] Consistent with this observation, at the same sampling time, the second most abundant genus was *Burkholderia*, which also have been found to aerobically degrade polycyclic aromatic hydrocarbons in oil-contaminated soil.[74] These shifts matched our observation of a large fraction of residual drilling fluid components and condensate oil at the beginning of the well 2 flowback (the image of condensate oil in the FPW samples is presented in Figure

S4). The large amount of oil fraction observed to be associated with well 2 FPW is likely because of the sampling location, which the well 2 samples were collected before the oil/water separator.

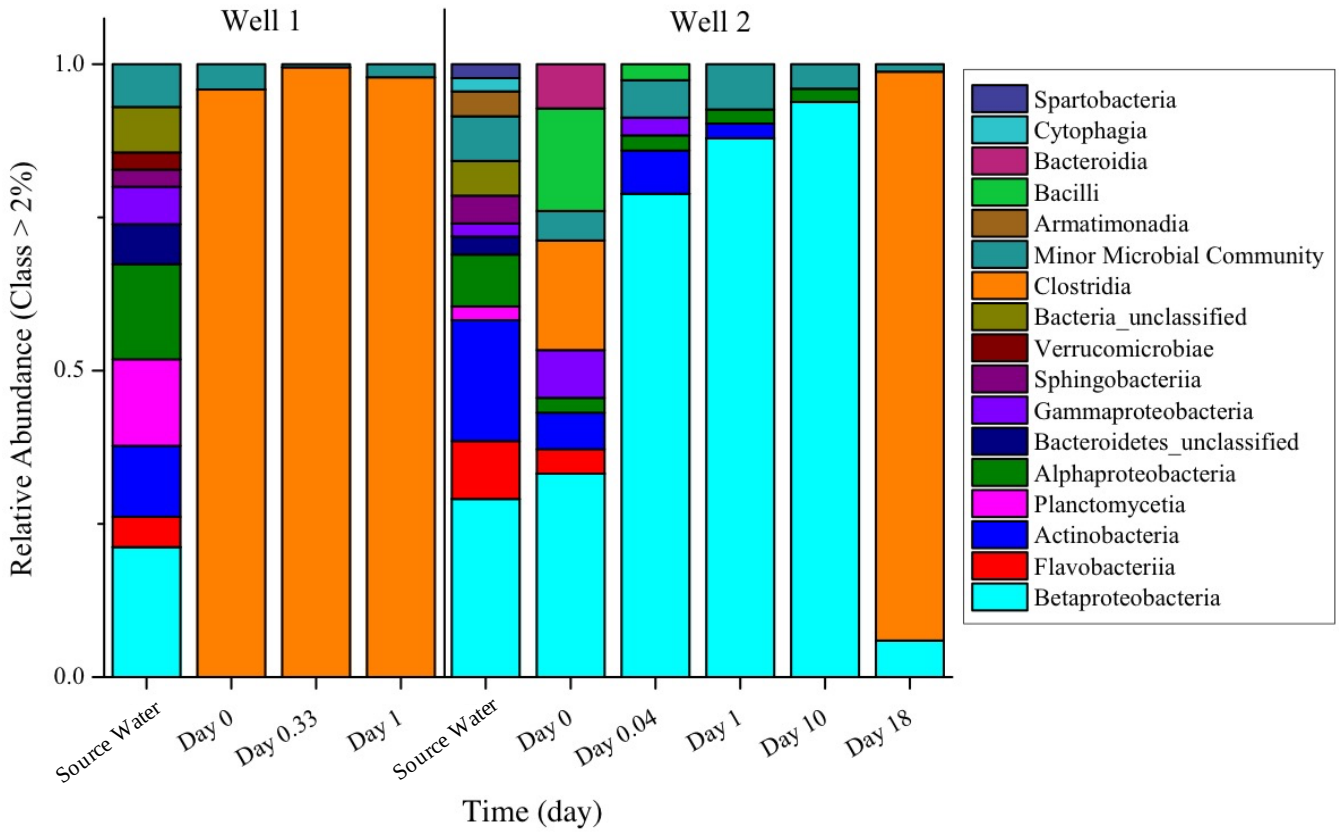

Figure 4 Class-level taxonomy of sequences obtained from source water and FPW for well 1 and well 2

~~**Figure 5** Maximum likelihood phylogenetic tree of *Halanaerobium* spp. 16S rRNA genes, sequences found in oil and gas environments are in red and those from fracturing sites are in red and bold (the complete tree is presented in Figure S2).~~

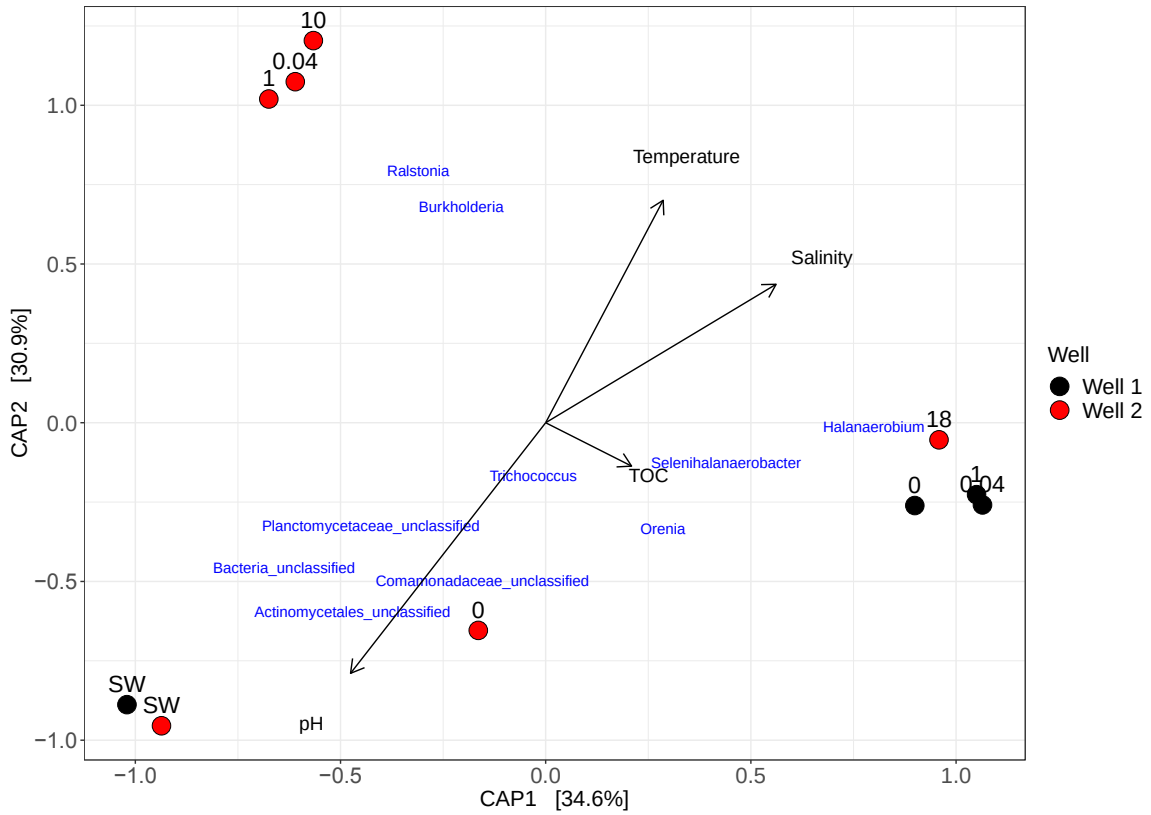

Figure 5 Similarity of microbial community between each sample shown on a CAP plot; the sample point labels indicate days since flowback initiation and arrows indicate the correlation between environmental variables and microbial community shifts

3.5 Biocide usage versus the subsurface selective forces

Given that strains of *Halanaerobium*, which may produce sulfide, are prevalent in many hydraulic fracturing sites, their presence in FPW highlights potential risks for unconventional resources extraction and the environment, such as facility corrosion, gas souring, and toxic gas generation.[61,63] The necessity of using biocides in high temperature formations to inhibit undesirable microbial activities has been previously discussed in the study of the Bakken Formation.[49] In this study, the biocide was found to be ineffective in inhibiting the enrichment of the sulfidogenic bacteria as the typical example of the genus

Halanaerobium, and as relatively higher cellular biomass and cell viability was observed during the early-stage of flowback. The injected biocide concentrations decreased likely through dilution by formation water and subsurface degradation. The quantification of the biocide ADBAC from well 2 FPW samples show that the biocide concentration decreased by over 50 times from samples collected at 1.44 hours to just before day 3, where it was then below the detection limit (<0.5 ppb).[27] There is limited knowledge on the biological resistance of *Halanaerobium* strains to ADBAC. However, the concentration of ADBAC detected in FPW was dramatically lower than the minimum inhibitory concentrations of the other common biocides such as quaternary ammonium compounds (13.5 mg/L) and glutaraldehyde (100 mg/L) on *Halanaerobium* DL-01 at laboratory conditions.[22] The rapid reduction in biocide concentration indicates that for the majority of the well flowback period, the dose of biocide is likely insufficient to inhibit microbial growth, as demonstrated by the relative enrichment of *Halanaerobium*. The chemical additives usually differs between operators, however the microbial communities across several formations constantly shifted toward *Halanaerobium*,[31,34] and other minor halotolerant/halophilic bacteria. This indicates that different hydraulic fracturing fluid formulas may have little influence on the long-term impact on the shift of microbial communities. Rather, the growth of thermophilic and halophilic bacteria suggests that subsurface conditions drive selection, including oxygen content, temperature and salinity. For this reason, a better understanding of reservoir geology will help to predict how microbial communities develop in the subsurface and, in turn, be beneficial to optimizing hydraulic fracturing technologies and hydraulic fracturing fluid formulas. Even though the biomass downhole appears low ($<10^5$ cells/mL) in the Duvernay shale reservoirs, the microbial communities shift to a few

key bacteria with potentially undesirable impacts. However, the 16S rRNA sequencing conducted in this study does not allow for the inference of microbial functions. Sequencing technologies such as metagenomic sequencing will be needed to further investigate the metabolic potentials of microbes in FPW from the Duvernay Formation. Undesirable gas monitoring showed the H_2S concentrations were below the detection limit for the two investigated wells (monitoring time was from 2016-09-09 to 2016-09-15 for well 1 and from 2016-12-05 to 2016-12-20 for well 2). Thus, continued monitoring on well performance is needed in the future.

4. Conclusions

Our study supports the view that *Halanaerobium*, a potentially sulfidogenic moderate-thermophilic halophile, is highly enriched in FPW in hydraulically fractured systems and rapidly becomes the dominant organism in subsurface systems. Lower abundances of methanogenic Archaea were observed relative to Bacteria, comprising less than 3% of total reads. .In the Duvernay play, DNA and cellular biomass are eventually reduced to below quantification limits of methods applied in this study by the factors including high salinity and temperature of the geological reservoir. Moreover, by directly comparing two wells drilled into the same formation, we show that recycling of FPW in one of the wells (well 1) enhances the enrichment and growth of this organism, allowing it to dominate the subsurface samples earlier in the process, and thereby may increase the rate of undesired side effects. Therefore, cross well contamination should be considered in the strategy of recycling of FPW, which is regarded as promising solution to reduce the water withdrawals from freshwater bodies in the process of making hydraulic fracturing fluid.

The efficiency of biocides in the downhole environment is limited, and in the wells studied here, they are ineffective in suppressing microorganisms in the first few days of flowback for both wells. Given that this study shows that *in situ* geological conditions, such as flowback salinity, play more critical roles in determining the microbial community structure, we encourage better assessments of the predicted downhole geochemical environments and optimize the use of biocides to achieve a more economic and environmentally sustainable selection of biocide type and amounts in making up hydraulic fracturing fluids.

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Supporting Information

Temporal Changes in Microbial Community Composition and Geochemistry in Flowback and Produced Water from the Duvernay Formation

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The supporting information contains the following:

(1) Detailed methods used to conduct cations and bromide.

(2) Detailed methods used to conduct untargeted organic analyses.

(3) Supplementary information for DNA extraction.

(4) Detailed methods to build the phylogenetic tree.

(5) Additional results for the organic analyses.

(6) The cross-section of the Duvernay Formation (Figure S1).

(7) The extracted ion chromatograms of ADBAC-C8, C10, C12 and C14 (Figure S2a), and the MS/MS spectrum of ADBAC-C8 ion at m/z 248.2372 under CID 25eV (Figure S2b).

(8) Maximum likelihood phylogenetic tree of *Halanaerobium* spp. 16S rRNA genes (Figure S3).

(9) Image showing condensate oil on the top of flowback and produced water (FPW) (Figure S4).

(10) Collection time for FPW samples after flowback commenced of well 1 and well 2 (Table S1).

(11) Temporal change in pH values of FPW after the initial FPW commenced from well 1 and well 2 (Table S2)

(12) Major inorganic components for well 1 source water and FPW (Table S3).

(13) Total cell numbers and cell viability for source water and FPW (Table S4).

(14) Spearman correlation between top 20 operational taxonomic units (OTU) and the Constrained Analysis of Principal Coordinates (CAP) ordination (Table S5).

1. Materials and Methods

1.1 Analysis of cations and bromide

For cation, total sulfur, and bromide analyses, samples were diluted using 18 MΩ ultrapure water by dilution factors of 700 for Na and 72 for all other element to match TDS requirements of the analytical instrument. Samples were acidified with 6 µL per 10 mL using 15.7 M trace metal grade nitric acid. All analyses for cations total sulfur, and bromide were performed using an Agilent 8800 Triple Quadrupole ICP-MS (ICP-QQQ) with a RF power of 1550 W and a RF reflected power of 18 W. The ICP-QQQ was operated with a microMist nebulizer and nickel/copper cones. Analyses were performed in high matrix introduction mode, MS/MS mode for greater mass resolution, and a gas collision/reaction cell with O_2 gas (10%), He (3 mL/min) and H_2 (5 mL/min) gas. An inline internal standard system was employed to add a solution of 0.5 ppm indium to each sample, which was used to correct for instrumental drift. Additionally, a standard solution of known concentration was analyzed at the beginning, middle and end of the run as an additional quality assurance (QA) / quality control (QC) check.

1.2 HPLC-Orbitrap MS analysis of organic compounds

50 µL of filtered flowback and produced water (FPW) was injected on a Hypersil Gold C18 analytical column (50 × 2.1 mm, 1.9 µm particle size; Thermo Fisher Scientific). The mobile phase at 0.4 mL/min was composed of (A) 15 mM ammonium acetate in water and (B) methanol. The gradient was as follows: 0-5 min, 1% B; 5-35 min, linear gradient to 100% B; 35-37 min, linear gradient back to 1% B and hold for 5 min. The first 4 min of eluent was discarded through a six port, two position valve, due to the high salinity of

most samples. The Orbitrap-MS was operated with an ESI source in positive ion mode (ESI^+). The ionization potential was set at 4 kV, while the sheath, aux, and sweep gas flows were set to 35, 15 and 2 (arbitrary units), respectively. Vaporizer and capillary temperature were at 325 °C and 300 °C, respectively. Acquisition was performed in full scan mode (*m/z* 100 to 1000) at 2.3 Hz with resolving power set to a nominal value of 120,000 at full width half-maximum at *m/z* 400. The tandem mass spectrometry analyses were performed using data dependent mode with collision-induced dissociation (CID) at 20, 25 and 35 eV, as well as by higher-energy collision dissociation (HCD) at 50 eV and 80 eV. Thermo Xcalibur 2.2 software was used for data acquisition and analysis. Reference standards were used for the identification of PEG, OPE and ADBAC through the comparison of retention time, mass spectra and tandem mass spectra. The external calibration curve of the reference standard ADBAC (C8-C14) spiked in HPLC grade water was used for semi-quantification of ADBAC in the FPW samples.

1.3 DNA extraction

For source water and FPW samples that had low DNA concentrations, 500 mL of each water sample was first filtered through 0.22 µm hydrophilic polypropylene membranes (GE Healthcare Life Sciences, Ontario, Canada), prior to DNA extraction and PCR amplification. If no PCR product band was observed after gel electrophoresis, filtrations of 1-2 L aliquots were done and the DNA extracted again. If DNA was not recovered following this procedure and after an additional duplicate extraction was performed, the total DNA concentration was regarded as below the detection limit.

1.4 Maximum likelihood phylogenetic tree of *Halanaerobium* spp. 16S rRNA genes

Majority consensus sequences were constructed for OTU2 and OTU3 (the two represented sequences related to *Halanaerobium* in this study) in Geneious R10, and the 50 top matches for each consensus sequence were retrieved by performing BLASTN searches on the National Center for Biotechnology Information database. In addition, we included the sequences of *Halocella cellulosilytica* (NR 036959.1) and *Halanaerobium salsuginis* (NR 044637.2) to provide a closely related out-group. Sequences were aligned using MAFFT v7.388 [1,2] within Geneious R10. All gaps were removed, and in cases where several sequences from the same study were identical in the aligned region, only one representative was kept. The phylogenetic tree was reconstructed using maximum likelihood method (GTR + Γ + I substitution model; shape parameter estimated; four rate categories) as implemented in PHYML3 [3] within Geneious R10. Bootstrapping analysis was performed with 100 replicates. Bootstrap support values below 50% are not shown. Sequence from isolates are shown with species names while only the Genbank accession number is included from sequences amplified from environmental samples. Origin of the sequences is included where available.

2. Results

2.1 HPLC-Orbitrap MS Analysis of Organic Compounds in FPW

For PEG and OPE homologues, the accurate mass measurement (mass error < 2 ppm) of protonated, ammonium adducts and sodium adducted ions, together with MS/MS spectra were used for identification, which was later confirmed by the corresponding reference standards. The PEG and OPE homologues were identified in our previous

study, please refer to He et al. for detailed information.[4] The extracted ion chromatography of ADBAC-C8 ($C_{17}H_{30}N^+$), ADBAC-C10 ($C_{19}H_{34}N^+$), ADBAC-C12 ($C_{21}H_{38}N^+$), ADBAC-C14 ($C_{23}H_{42}N^+$) (Figure S2a) showed the increased retention time of these homologues on the C18 column used, which is consistent with the increased chain length from C8 to C14. The MS/MS spectrum of $C_{17}H_{30}N^+$ at *m/z* 248.2372 (Figure S2b) showed the product ion $C_{10}H_{22}N^+$ at *m/z* 156.1744 from the fragmentation at benzylic amine bond and also the tropylium ion $C_7H_7^+$ at *m/z* 91.0539, supporting the structural identification of ADBAC-C8. The same fragmentation pattern of ADBAC was also observed in another study.[5] MS/MS analysis was also performed on a commercially available standard ADBAC-C8, which further confirmed our identification.

3. Figures

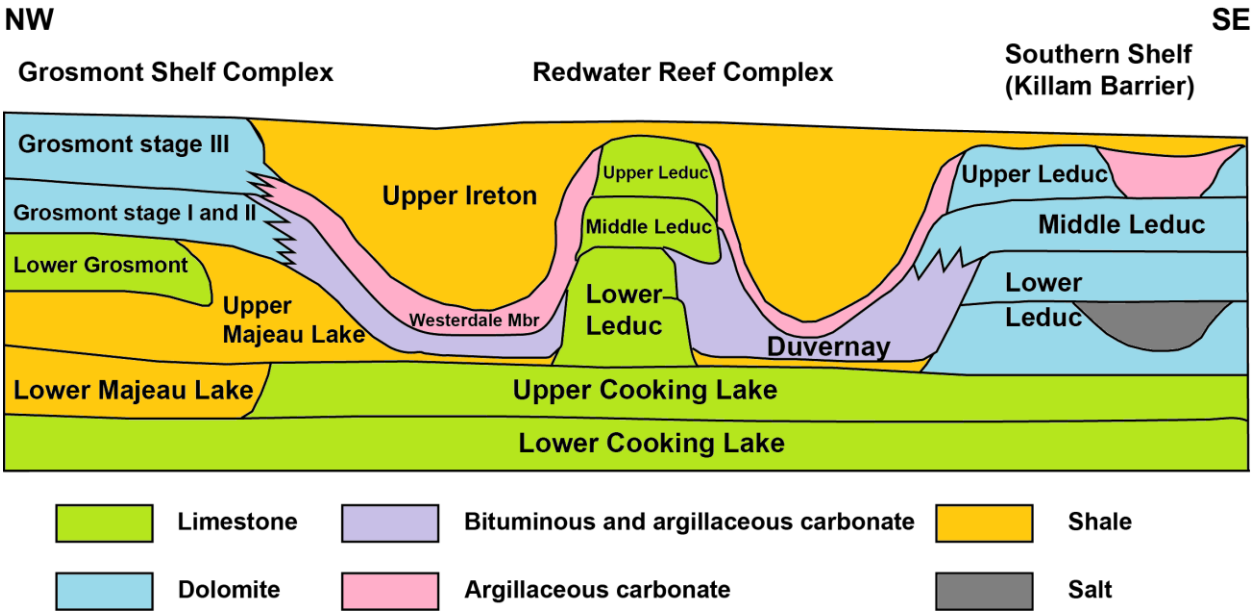

Figure S1 Adapted schematic cross-section of Leduc reef and basinal deposits including Duvernay Formation during the Devonian in Alberta, Canada.[6]

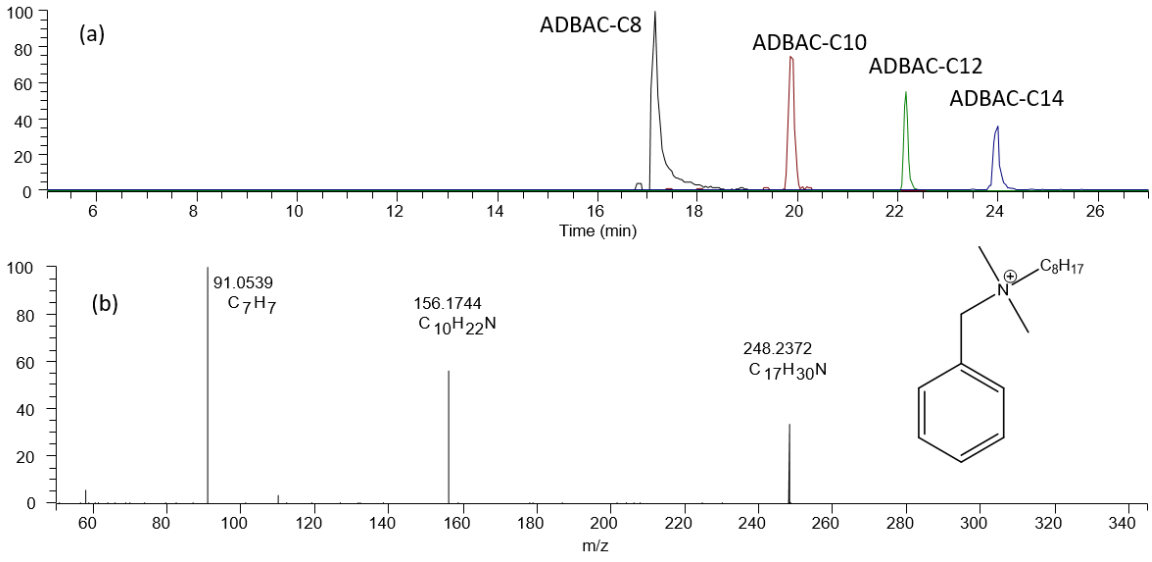

Figure S2 (a) Extracted ion chromatograms of (a) ADBAC-C8, C10, C12 and C14, and (b) the MS/MS spectrum of ADBAC-C8 ion at *m/z* 248.2372 under CID 25eV. The structure of ADBAC-C8 is also illustrated in caption (b).

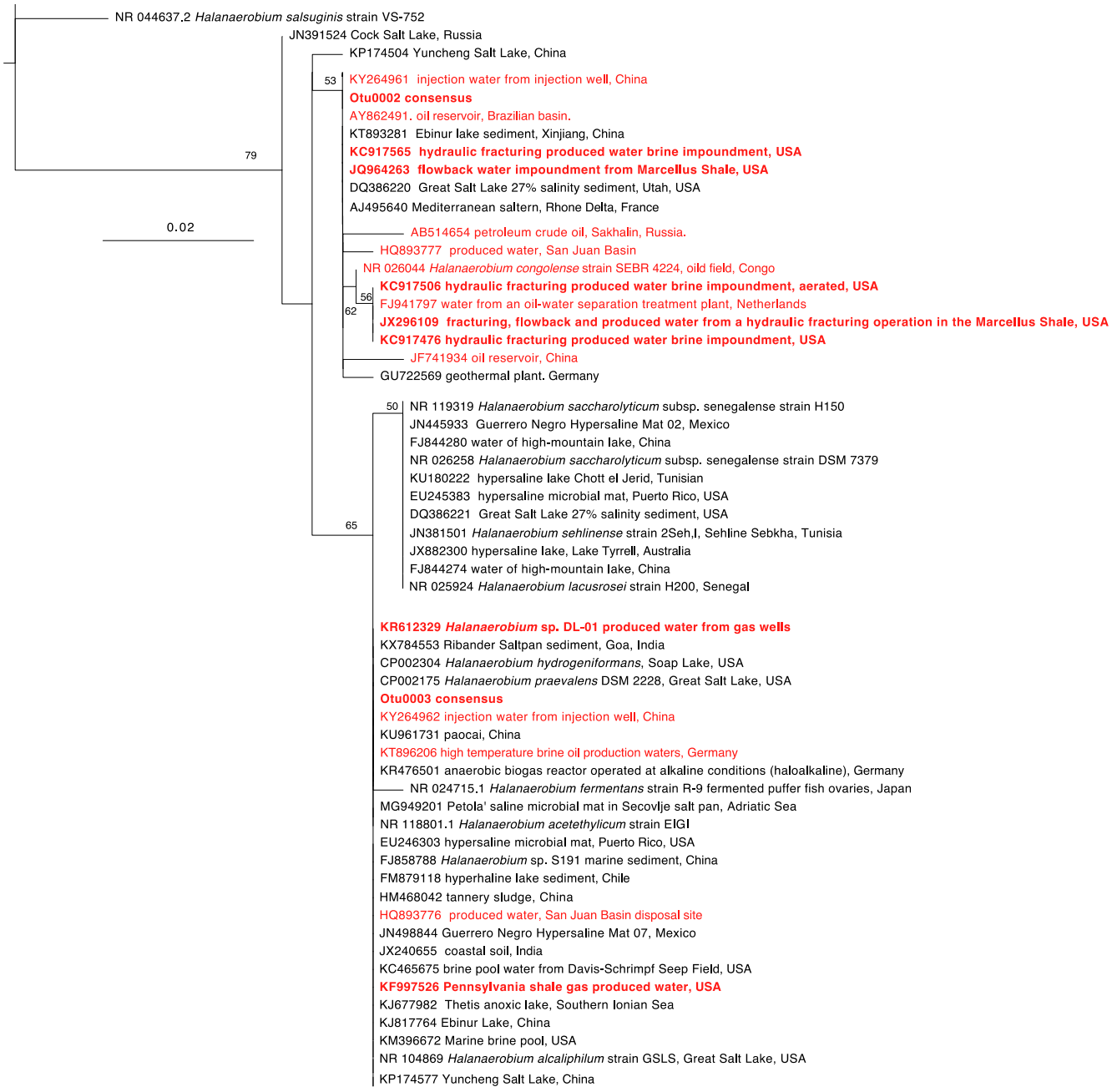

Figure S3 Maximum likelihood phylogenetic tree of *Halanaerobium* spp. 16S rRNA genes, sequences found in oil and gas environments are in red and those from fracturing sites are in red and bold.

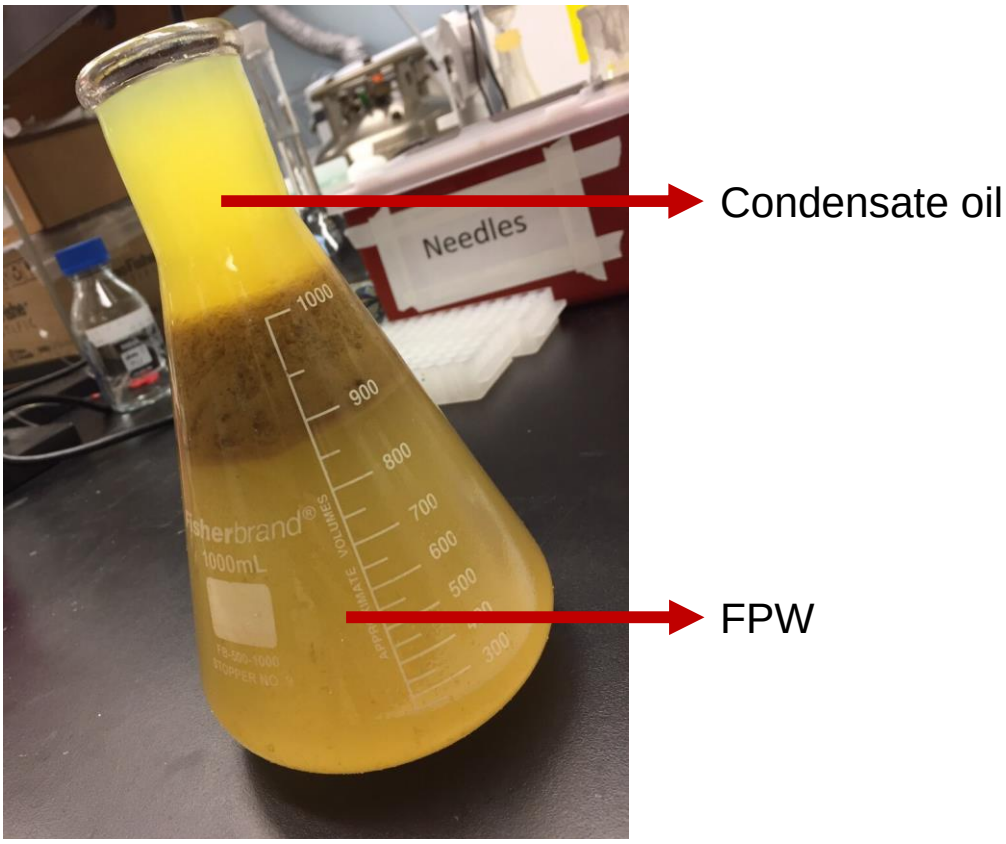

Figure S4 Image showing condensate oil on the top of FPW.

4. Tables

Table S1 Collection time for FPW samples (for microbiology study) after flowback commenced of well 1 and well 2 since 2016-09-09 and 2016-12-05, respectively

Well	Type	Time Post Initial Flowback
Well 1	Source Water	
	FPW	Day 0
		Day 0-8 h
		Day 1
		Day 2
		Day 3
		Day 4
		Day 5
		Day 6
		Day 19
		Day 20
		Day 32
		Day 53
		Day 91
		Day 120
well 2	Source Water	
	FPW	Day 0
		Day 0-1 h
		Day 0-2 h
		Day 0-3 h
		Day 0-5 h
		Day 0-8 h
		Day 0-16 h
		Day 1
		Day 1-8 h
		Day 2
		Day 2-8 h
		Day 3
		Day 4
		Day 5
		Day 9
		Day 10
		Day 11

		Day 12
		Day 13
		Day 14
		Day 15
		Day 18
		Day 34
		Day 40
		Day 102

Table S2 Temporal change in pH values of FPW after the initial FPW commenced from well 1 and well 2

Time (Day)	Well 1	Well 2
Source Water	8.1	7.4
Recycled Produced Water	4.7	
0	6.8	7.6
0.08		5.4
0.13		5.4
0.21		5.4
0.33	5.4	5.8
0.67	5.5	5.2
1	5.5	5.5
1.33	5.4	
1.67	5.4	
2	5.4	5.5
2.33	5.3	5.9
2.67	5.4	
3	5.3	
3.33	5.3	
3.67	5.3	
4	5.3	6.0
4.33	5.3	
4.67	5.3	
5	5.5	
5.33	5.7	
5.67	5.8	
6	5.6	
9		5.6

10		5.52
12		5.56
15		5.47
18		5.5
19	6.11	

Table S3 Major inorganic components for well 1 source water and FPW, parts per million (ppm)

Well 1										
Time (Day)	Na (ppm)	B (ppm)	Mg (ppm)	K (ppm)	Ca (ppm)	Fe (ppm)	Sr (ppm)	Br (ppm)	Cl (ppm)	S (ppm)
Source Water	10	BDL	7	1	33	BDL	BDL	BDL	4	2
0	1397	3	24	52	198	BDL	19	6	3108	6
0.33	54510	77	738	1930	8799	135	893	237	115284	80
0.67	53596	79	731	1924	8685	151	893	240	113656	82
1	54257	79	738	1929	8626	151	904	242	111908	78
2	56822	81	768	2031	8503	50	947	246	126340	68
3	56368	83	806	2098	8976	84	1009	257	126539	67
4	55374	81	814	2121	9052	76	1037	270	130501	63
5	55605	78	830	2005	9762	58	1049	350	NA	80
6	56164	79	835	1986	9604	53	1067	277	NA	74
19	60315	82	923	2098	10771	NA	1228	310	NA	69
32	65164	82	808	2077	11333	NA	1234	319	NA	NA
53	67762	90	853	2163	11567	925	1290	331	NA	40
91	56859	80	869	2066	10094	140	985	259	119780	40
Well 2										
Time (Day)	Na (ppm)	B (ppm)	Mg (ppm)	K (ppm)	Ca (ppm)	Fe (ppm)	Sr (ppm)	Br (ppm)	Cl (ppm)	S (ppm)
0.06	47382	67	614	1574	6759	185	673	228	83882	87
0.08	44181	69	579	1469	6210	154	632	220	81964	84
0.13	47484	75	657	1598	6936	167	724	260	73666	100
0.21	46199	72	629	1536	6685	167	685	236	70758	100
0.33	44577	73	623	1530	6184	73	679	233	NA	94
0.67	47878	74	666	1613	6984	107	725	246	85910	102

1	46812	73	674	1615	7163	69	741	251	NA	98
2	50734	79	718	1678	7292	65	787	264	94091	97
3	50249	76	744	1735	7718	54	827	270	81424	102
4	50489	76	737	1691	7818	61	822	269	NA	90
5	52030	79	776	1760	8011	70	867	271	NA	89
5.67	46707	85	831	1880	8530	85	939	299	95106	90
9.38	53656	68	793	1976	8875	81	864	259	107222	NA
9.5	54122	85	800	1941	9058	68	881	231	103053	62
10	55445	88	831	2016	9646	58	917	260	NA	NA
10.83	55349	85	823	1988	9890	43	907	254	94962	59
11.75	56844	74	835	2106	9718	53	926	245	102534	NA
12.08	56632	84	837	2022	9750	21	922	262	NA	58
13.5	58836	80	869	2097	9711	78	976	254	99948	59
14.08	57872	80	866	2088	10217	73	974	257	NA	62
17.5	66263	90	989	2634	12989	100	1307	312	134487	32
30.5	NA	NA	NA	NA	NA	NA	NA	NA	148066	NA
36.5	NA	NA	NA	NA	NA	NA	NA	NA	101155	NA

Note: below detection limit (BDL), not available (NA)

Table S4 Total cell numbers and cell viability for source water and FPW; counts below the detection limit of 10^5 cells mL^{-1} (<200 cells in total 15 microscope fields) in red.

Well	Type	Time (Day)	Total Cell Numbers (10^6 cells mL^{-1})	Cell Viability (%)
Well 1	Source Water		2.58±0.10	46
	FPW	0	0.79±0.19	38
		0.33	0.72±0.13	20
		1	0.15±0.11	20
		2	0.15±0.07	7
		3	0.25±0.14	41
		4	0.09±0.09	25
		5	0.06±0.05	28
		6	0.08±0.08	22
		19	0.1±0.04	58
		20	0.07±0.04	47
		32	0.09±0.07	48
		52	0.03±0.02	9
		91	0.04±0.05	8
Well 2	Source Water		1.04±0.30	23
	FPW	0	0.48±0.14	40
		0.04	0.72±0.19	43
		1	0.40±0.29	35
		10	0.10±0.07	12
		18	0.13±0.07	33
		34	0.09±0.12	35
		40	0.05±0.04	42
		102	0.11±0.13	8

Table S5 Spearman correlation between top 20 operational taxonomic units (OTU) and the CAP ordination

***VECTORS								
	CAP1	CAP2	r2	Pr(>r)				
Halanaerobium	0.9964	-0.08477	0.9092	0.001	***			
Bacteria_unclassified	-0.727	-0.68664	0.9041	0.001	***			
Ralstonia	-0.33068	0.94374	0.9954	0.001	***			
Flavobacterium	-0.67865	-0.73446	0.7931	0.005	**			
Actinomycetales_unclassified	-0.66828	-0.74391	0.834	0.001	***			
Planctomycetaceae_unclassified	-0.71022	-0.70398	0.4551	0.005	**			
Comamonadaceae_unclassified	-0.58832	-0.80863	0.4974	0.067	.			
Burkholderiales_unclassified	-0.67917	-0.73398	0.8191	0.004	**			
Betaproteobacteria_unclassified	-0.71499	-0.69914	0.8088	0.001	***			
Bacteroidetes_unclassified	-0.69743	-0.71665	0.6972	0.005	**			
Selenihalanaerobacter	0.98731	-0.15883	0.3938	0.133				
Polynucleobacter	-0.65177	-0.75842	0.9116	0.002	**			
Burkholderia	-0.32798	0.94468	0.9557	0.003	**			
Orenia	0.758	-0.65225	0.3069	0.271				
Actinobacteria_unclassified	-0.67578	-0.73711	0.6163	0.005	**			
Aquabacterium	-0.46541	-0.88509	0.1433	0.777				
Propionibacterium	-0.37739	0.92605	0.3722	0.109				
Trichococcus	-0.33649	-0.94169	0.085	0.988				
Pelomonas	-0.32282	0.94646	0.7338	0.007	**			
Bacteroides	-0.30419	-0.95261	0.0912	0.946				

Signif.	codes:	0	‘***’	0.001	‘**’	0.01	‘*’	0.05
Permutation:	free							
Number	of	permutation	999					

Reference

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